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CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Novel Proteolytic Microvesicles Released from Human Macrophages after Exposure to Tobacco Smoke

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Cigarette smoking damages the extracellular matrix in a variety of locations, leading to atherosclerotic plaque instability and emphysematous lung destruction, but the underlying mechanisms remain poorly understood. Here, we sought to determine whether exposure of human macrophages, a key participant in extracellular matrix damage, to tobacco smoke extract (TSE) induces the release of microvesicles (MVs; or microparticles) with proteolytic activity; the major proteases involved; and the cellular mechanisms that might mediate their generation. We found that MVs released from TSE-exposed macrophages carry substantial gelatinolytic and collagenolytic activities that surprisingly can be predominantly attributed to a single transmembrane protease of the matrix metalloproteinase (MMP) superfamily (namely, MMP14). Flow cytometric counts revealed that exposure of human macrophages to TSE for 20 hours more than quadrupled their production of MMP14-positive MVs (control, 1112 ± 231; TSE-induced, 5823 ± 2192 MMP14-positive MVs/μL of conditioned medium; means ± SEM; $n = 6$; $P < 0.01$). Our results indicate that the production of these MVs by human macrophages relies on a series of regulated steps that include activation of two mitogen-activated protein kinases (MAPKs, i.e., the Jun N-terminal kinase and p38 MAPK), and then MAPK-dependent induction and maturation of cellular MMP14, a remarkable accumulation of MMP14 into nascent plasma membrane blebs, and finally caspase- and MAPK-dependent apoptosis and apoptotic microvesicle generation. Proteolytically active MVs induced by tobacco smoke may be novel mediators of clinical important matrix destruction in smokers. *(Am J Pathol 2013, 182: 1552–1562; http://dx.doi.org/10.1016/j.ajpath.2013.01.035)*

Microvesicles (MVs), also known as microparticles, are small membranous structures that are released from cells during activation or apoptosis.[1–7] Recent studies from our laboratory,[5] as well as other groups,[8,9] have shown that exposure to tobacco smoke, an important risk factor for atherosclerosis and respiratory diseases, increases MV generation from cultured human cells *in vitro*[5] and in the circulation of humans *in vivo*.[8,9]

The potential pathophysiological involvement of MVs in different diseases has attracted considerable attention in the past few years.[1–7] Macrophages play key roles in the destabilization and rupture of atherosclerotic plaque[10–12] and in emphysematous lung destruction[13,14] through their expression of a variety of proteases.[11,13,15] It has not been previously explored whether MVs released from human macrophages after tobacco smoke exposure can cause collagen degradation, thereby contributing to extracellular

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matrix damage in the cardiovascular and respiratory systems in smokers.

In the current study, we sought to determine whether exposure of human macrophages to tobacco smoke extract (TSE) induces the release of MVs with proteolytic activity, what the nature of the major proteases on smoke-induced macrophage MVs might be, and what cellular mechanisms might be responsible for their generation. Given the vesicular structure of MVs, three general types of proteases appeared possible: i) transmembrane,[16–18] ii) intracellular,[19] and iii) non-transmembrane but surface-bound.[20] Surprisingly, we found that smoke-induced macrophage MVs carry significant gelatinolytic and collagenolytic activities that could be predominantly attributed to a single transmembrane protease of the matrix metalloproteinase (MMP) superfamily (ie, MMP14), which is also known as the membrane type 1 MMP.[16,21,22]

Materials and Methods

Reagents and Antibodies

PhosphoPlus antibody kits against three major mitogen-activated protein kinases (MAPK): i) Jun N-terminal kinase (JNK) (phospho-Thr183/Tyr185, catalog #9250), ii) p38 MAPK (phospho-Thr180/Tyr182, catalog #9211), and iii) extracellular signal-regulated kinase (ERK) (phospho-Thr202, catalog #9100) were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies against human MMP14 were purchased from Abcam (Cambridge, MA) or R&D Systems (Minneapolis, MN). Phycoerythrin-labeled annexin-V was purchased from BD Pharmingen (San Jose, CA). Inhibitors of JNK (SP600125, catalog #5567), p38 (SB202190, catalog #7067), and ERK (U0126, catalog #U120) were obtained from Sigma-Aldrich (St. Louis, MO). The pan-caspase inhibitor (Z-VAD-FMK) was purchased from R&D Systems. Gelatin from porcine skin (119K0062) and native collagen from human placenta (C7774) were purchased from Sigma-Aldrich.

Preparation of Tobacco Smoke Extract

Research-grade cigarettes were obtained from the Reference Cigarette Program at the University of Kentucky (Lexington, KY). Full-strength stock tobacco smoke extract (TSE, 100%) was prepared by extracting mainstream smoke from four cigarettes through 10 mL of phenol red-free RPMI 1640 medium containing 0.2% bovine serum albumin (BSA), at one cigarette per 7 to 8 minutes, simulating the burning rate of typical smoking.[5,23–26] We followed our previous method,[5] which was modified here to improve extraction efficiency by using a Kontes gas washing bottle (Kimble/Kontes Glass Co., Vineland, NJ) that allows the system to be sealed and increases the travel distance for smoke bubbles through the medium. Stock preparations of TSE were adjusted to a pH of 7.4 and then sterilized by passage through a filter with a 0.22-μm size cut-off. Our initial studies indicated that a single freeze-thaw cycle did not alter the biological effects of TSE on cultured cells, and therefore TSE was aliquoted and stored at −80°C. To ensure consistency among preparations, each batch of TSE was standardized according to its absorbance at 320 nm[5,27,28] and re-standardized to its potency to induce apoptosis of THP-1 monocytic cells[5] by comparison with aliquots of a single standard preparation that was made at the beginning of the study.

Cell Culture and Differentiation

Human THP-1 monocytic cells were differentiated into macrophage-like cells (called THP-1 macrophages)[4,5] (ATCC) by transient exposure to phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) by following previously described methods with minor modifications.[29,30] Briefly, THP-1 monocytic cells were differentiated with 50 ng/mL PMA for 48 hours. The resulting adherent cultured THP-1 macrophages were rinsed with warmed phosphate-buffered saline (PBS), followed by 48 hours wash-out incubation in fresh RPMI 1640/10% fetal bovine serum, without PMA, to minimize ongoing effects of this compound. Primary human monocyte-derived macrophages (hMDMs) were prepared from fresh buffy coats by selecting monocytes via plate adherence, followed by differentiation into macrophages as described.[4,5,31] These two cell types are commonly used as models of the macrophages in atheromata[4,5,32] and in pulmonary alveoli.[33–36]

At the beginning of each experiment, THP-1 macrophages or primary hMDMs were transferred to serum-free RPMI 1640 medium/BSA supplemented with different concentrations of TSE, ranging from 0% (control) to 2.5% (v:v, the highest concentration being approximately equivalent to a 60 kg person smoking 2 packs of cigarettes), and then incubated at 37°C for 0 to 20 hours. In time-course studies, all groups of cells were simultaneously placed into serum-free medium and simultaneously harvested; TSE was added at indicated times before harvest. In experiments using Z-VAD-FMK or MAPK inhibitors, the compounds were added to cells 1 hour before the addition of TSE and remained until the end of the study, at concentrations of 50 μmol/L Z-VAD-FMK, 10 μmol/L SP600125, 10 μmol/L SB202190, and 10 μmol/L U0126.

Isolation and Immunoblotting of Cultured Cells and Their MVs

After treatment of THP-1 macrophages or hMDMs with or without TSE, the conditioned medium was harvested and centrifuged twice at 1500 × *g* for 10 minutes to eliminate cell debris and then at 100,000 × *g* for 30 minutes at 4°C to pellet MVs. The MVs were then washed by resuspension in PBS, then ultracentrifuged again, and the washed pellet was subsequently resuspended in fresh RPMI 1640 with 0.1% Triton X-100 for assessment of proteolytic activities or in cell lysis buffer for immunoblots. For immunoblots, cells and isolated MVs were lysed in a buffer containing 20 mmol/L

Tris/HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EGTA, 1% Triton X-100, 1% sodium deoxycholate, 1 mmol/L EDTA, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Na_3VO_4 (an inhibitor of protein tyrosine phosphatases), and a commercial protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany) for 30 minutes on ice. Samples (50 to 80 μg protein/lane) were electrophoresed through a 4% to 15% precast linear gradient polyacrylamide gel, followed by a transfer of proteins to nitrocellulose membranes and then immunoblotting, as described.[5] Bands were visualized using an enhanced chemiluminescence detection kit (Thermo Scientific, Waltham, MA). Signals were quantified using ImageJ version 1.45 densitometry software and normalized in each independent experiment to values from control cells.

Assays of Proteolytic Activities of Isolated MVs

We used three separate methods to assess the proteolytic activities of MVs from control and TSE-exposed cells. The first method used fluorogenic substrate I (ES001; R&D Systems), which harbors the consensus sequence Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 that is cleaved by MMP1, MMP2, MMP7, MMP8, MMP9, MMP12, MMP13, MMP14, MMP15, and MMP16. Cleavage of fluorogenic substrate I generates a fluorescent product. We pre-warmed isolated MVs and 10 μmol/L fluorogenic substrate I in separate aliquots of activity buffer (25 mmol/L Tris/HCl, pH 8.0, containing 2.5 μmol/L $ZnCl_2$, and 3 mmol/L $CaCl_2$) at 37°C for 0.5 hours. These aliquots were mixed to initiate the enzymatic reaction in a final volume of 100 μL within wells of a 96-well plate at 37°C. Cleavage of the substrate was kinetically monitored at 320-nm excitation and 405-nm emission every 20 minutes for 2 hours.

The second method we used to assess proteolytic activity in control and TSE-induced MVs was gelatin zymography, which was optimized using features taken from several published protocols.[16,37,38] In brief, we prepared 8% SDS-PAGE gels containing 1 mg/mL gelatin. Homogenates of isolated MVs were loaded onto these gels under nonreducing conditions, and were run at 100 V for 120 minutes with molecular weight standards (Lonza 50550, Walkersville, MD). Gels were then washed four times in zymogram renaturing buffer (LC2670; Invitrogen, Grand Island, NY) and incubated overnight in zymogram development buffer (LC2671; Invitrogen) at 37°C. Gels were then stained with 0.2% Coomassie Blue R-250 followed by two rounds of destaining in 55% methanol and 7% acetic acid. The gels were scanned for image capture and densitometric analysis using the Epson Perfection V700 Photo Flatbed Scanner (Long Beach, CA).

The third method to assess proteolytic activity in MVs was native collagen zymography, which we also optimized using features taken from several published protocols.[16,39,40] We made our native collagen gels by pouring them under controlled temperature and pH to avoid collagen denaturation. MV homogenates were loaded onto 8% SDS-PAGE gels containing 0.5 mg/mL of collagen under nonreducing conditions, and were then run and processed exactly the same as the gelatin zymograms.

Flow Cytometry

Flow cytometry of MVs and cells was performed according to our published protocols.[4,5] In brief, at the end of each incubation, culture supernatants were fixed by the addition of filtered paraformaldehyde to a final concentration of 1%, and then supplemented with latex beads as a reference for MV counts. The number of MVs in each sample was quantified by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ), using gating criteria based on particle size, as detected by forward scatter, and surface exposure of phosphatidylserine (PS), as detected by staining with PE-labeled annexin-V (BD Pharmingen).[4,5] The portion of these PS-positive MVs meeting standard size criteria that were also MMP14-positive was quantified by simultaneous staining with anti-human MMP14 monoclonal antibody (ab78738; Abcam), followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG secondary antibody (Abcam). Adherent monolayers of THP-1 macrophages and hMDMs were scraped and fixed by suspension in 1% paraformaldehyde and then 70% ethanol. Late-stage apoptosis was assessed by TUNEL (APO-DIRECT kit, BD Pharmingen) and was also quantified by flow cytometry.

Confocal Microscopy

For confocal fluorescence microscopy, adherent THP-1 macrophages were incubated without (control) or with 2.5% TSE for 20 hours at 37°C, and were then fixed on the plates with 4% paraformaldehyde in PBS on ice for 10 minutes. The fixed cells were then washed twice with PBS, followed by a blocking solution (1% BSA and 0.1% Triton X-100 in PBS) for 60 minutes at room temperature. The cells were simultaneously incubated with 10 μg/mL primary anti-human MMP14 antibody (Abcam) and 10 μL/mL PE-labeled annexin V, thereafter, in the presence of 1 mmol/L $CaCl_2$, in the dark at 4°C overnight. The cells were washed three times with 0.2% BSA in PBS to remove unbound primary antibody and annexin V, and then incubated with 1:2000 FITC-labeled goat anti-mouse IgG secondary antibody in PBS with 1% of BSA for 1 hour. The stained cells were analyzed with a Leica TCS SP5 fluorescent confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL).

Statistical Analysis

Normally distributed data are shown as means ± SEM (n = 4 to 6). Comparisons among three or more groups were performed using one-way analysis of variance followed by the Student-Newman-Keuls (SNK) test, with $P < 0.05$ considered significant. Comparisons between two groups used the Student's unpaired, two-tailed t-test. Comparisons between two groups of non-normally distributed data used the Mann-Whitney rank-sum test.

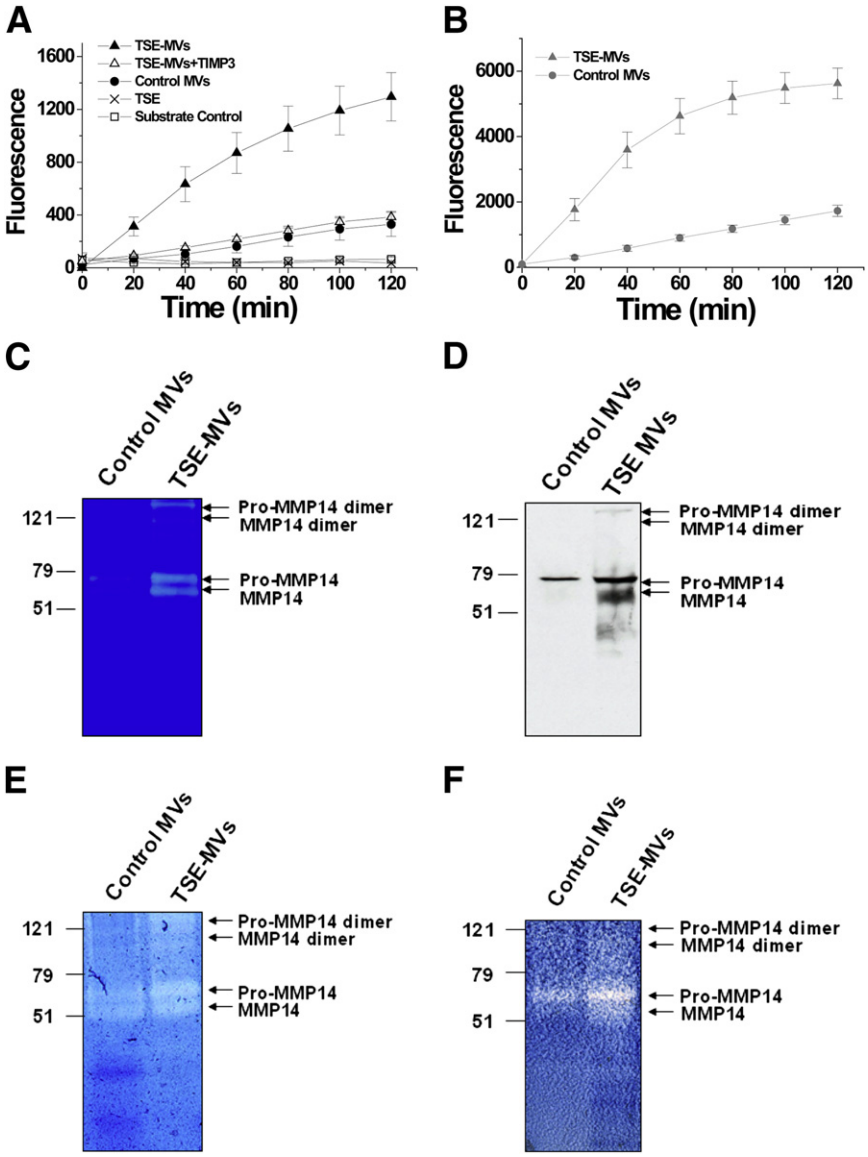

Figure 1 Exposure of human macrophages to tobacco smoke induces the release of potently proteolytic microvesicles in which MMP14 is the predominant gelatinase and collagenase. Microvesicles were purified from the conditioned media of human THP-1 macrophages (3×10^6 cells) (**A**, **C**, **D**, and **E**), and hMDMs (0.8×10^6 cells) (**B** and **F**) that had been incubated for 20 hours at 37°C in Dulbecco's modified Eagle's medium/BSA supplemented with buffer (Control MVs) or 2.5% TSE (TSE-MVs). **A** and **B**: Representative kinetic curves for the cleavage of fluorogenic substrate 1, a known substrate for MMPs, by control and TSE-induced MVs (TSE-MVs) from the two preparations of human macrophages. These curves indicate that our measurements were taken without saturation of the assay. For additional validation, we also tested fluorogenic substrate I without MVs (Substrate control), 2.5% TSE with no MVs (TSE), and TSE-MVs supplemented with 50 μmol/L tissue inhibitor of metalloproteinase 3 (TIMP3), a known inhibitor of MMPs (TSE-MVs+TIMP3) (**A**). $P < 0.001$ by analysis of variance at each time point after 20 minutes, but the only values significantly different from the other four groups came from TSE-MVs ($P < 0.05$, Student-Newman-Keuls test) (**A**). $P < 0.05$ by Student's *t*-test at each time point after 20 minutes (**B**). **C**—**F**: Human macrophage-derived MVs were extracted and then analyzed by gelatin zymography (**C**), and immunoblotting using anti-MMP14 antibodies (**D**) and native collagen zymography (**E** and **F**). Zymograms and immunoblots (shown in **C**—**F**) display images of full-length lanes representative of more than three independent experiments. Indicated are molecular-weight markers in kilodaltons, as well as pro-MMP14, mature MMP14 (*MMP14*), and their dimers. All other bands were faint. Dark flecks (shown in **E** and **F**) are focal precipitates of native collagen that form inadvertently when these gels cool and solidify after pouring.

Results

Exposure of Human Macrophages to Tobacco Smoke Induces the Release of Potently Proteolytic Microvesicles in Which MMP14 Is the Predominant Gelatinase and Collagenase

To determine whether exposure of human macrophages to tobacco smoke induces the release of MVs with proteolytic activity, we began by isolating MVs from the conditioned media of control and TSE-treated human THP-1 macrophages and primary hMDMs, and then examining the ability of these particles to cleave fluorogenic substrate I, a commonly used artificial substrate for MMPs. Based on the linear portion of the reaction curves, TSE-MVs showed striking proteolytic activity, reaching 3 times the values from control MVs (Figure 1, A and B). Our TSE preparation, which is washed away during isolation of MVs, contained no detectable protease activity (Figure 1A). Next, to determine the nature of the major proteases on smoke-induced MVs, we showed that their activity is nearly completely inhibited by the tissue inhibitor of metalloproteinase 3 (Figure 1A), indicating one or more MMPs. To examine activities against components of the extracellular matrix, we performed zymography of MV homogenates, using two relevant substrates — namely, gelatin (ie, partially hydrolyzed collagen) (Figure 1C) and native triple-helical collagen (Figure 1, E and F). Images of full-length lanes are displayed (Figure 1, C—F) to allow detection of all active enzymes. Surprisingly, we found significant gelatinolytic and collagenolytic activity carried by smoke-induced macrophage MVs that could be attributed predominantly to a single transmembrane protease of the MMP superfamily — namely, MMP14, the identity of which was confirmed by molecular weight from zymography (Figure 1, C, E, and F) and from immunoblotting with a specific monoclonal antibody against MMP14 (Figure 1D). The finding of collagenolytic activity strongly

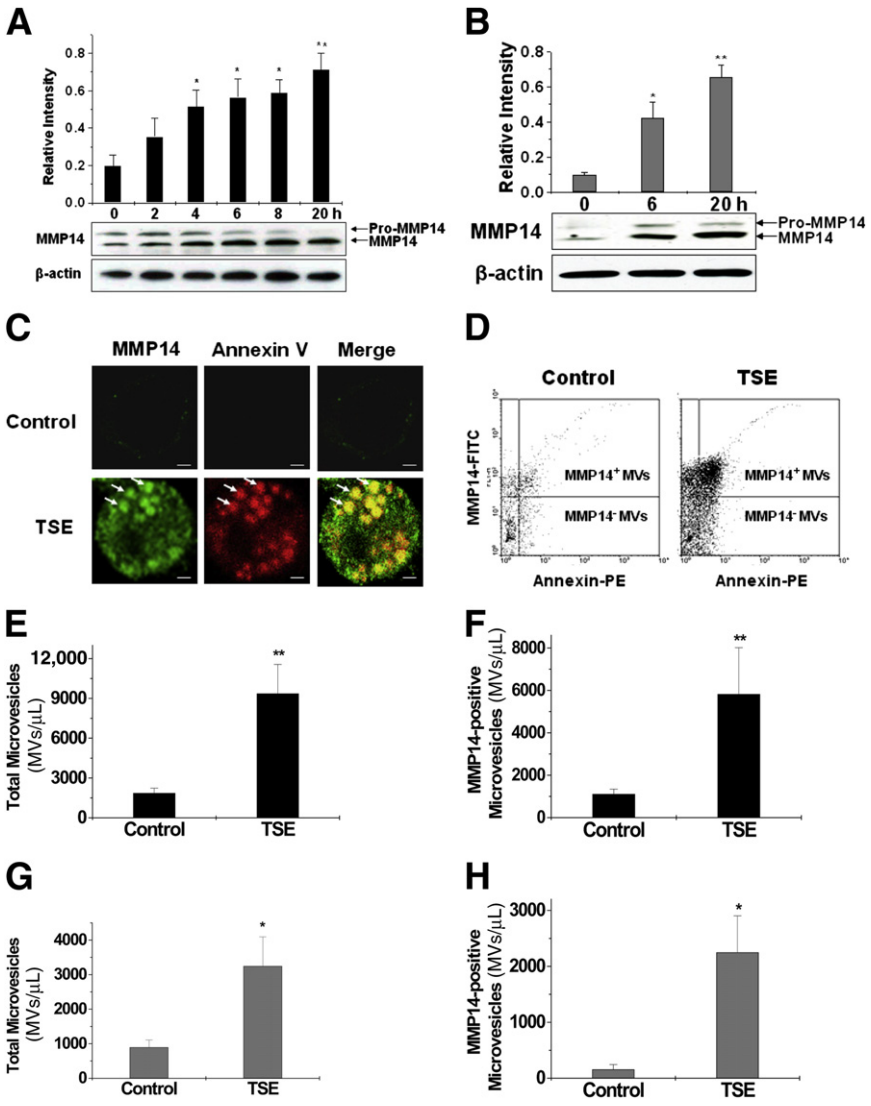

Figure 2 Exposure of human macrophages to TSE induces MMP14 expression, activation, accumulation in small circumscribed cell-surface domains, and release on microvesicles. **A** and **B**: Quantifications and representative immunoblots of total MMP14 content of human THP-1 macrophages (**A**) and primary hMDMs (**B**) after exposure to 2.5% TSE for 0 to 20 hours. All cells were harvested simultaneously; TSE was added at the indicated times before harvest. $P < 0.001$ by analysis of variance; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus values at 0 hours by the Student-Newman-Keuls post hoc test. **C**: Confocal fluorescent micrographs of representative THP-1 macrophages that were incubated without (Control) or with 2.5% TSE for 20 hours, as indicated, and then stained simultaneously with anti-human MMP14 antibodies (detected with green FITC-labeled secondary antibodies) and phycoerythrin-labeled annexin V (red, which preferentially binds exteriorized phosphatidylserine). Confocal slices were taken to visualize the upper surface of the cells. The yellow color in the merged images (Merge) demonstrates colocalization of the two labels. **Arrows** indicate examples of the small, circumscribed, cell-surface domains that stain intensely for both MMP14 and exteriorized PS and exhibit the same size as TSE-induced MVs, consistent with enrichment of MMP14 in nascent membrane blebs. Scale bar = 1 μm. **D**: MVs from control and TSE-treated THP-1 macrophages were analyzed by flow cytometry. Displayed are representative dot plots of staining by primary antibodies against MMP14 (detected with FITC-labeled secondary antibodies) versus annexin V-phycoerythrin staining (indicating surface PS exposure). The large **upper right rectangular regions** in each dot plot indicate the subset of MVs that are MMP14-positive ($MMP14^{+}$ MVs). The **lower-right rectangles** indicate MVs that did not meet our threshold for MMP14 staining ($MMP14^{-}$ MVs). **E**–**H**: Concentrations of total or MMP14-positive MVs in conditioned media from control and TSE-exposed THP-1 macrophages (**E** and **F**) and primary hMDMs (**G** and **H**), as assessed by flow cytometry. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control values by the unpaired two-tailed Student's *t*-test.

suggests a potential role for smoke-induced macrophage MVs in destabilizing atherosclerotic plaques[41–43] and damaging lung tissues[13,44–46] in smokers.

Exposure of Human Macrophages to TSE Induces MMP14 Expression, Activation, Accumulation in Small, Circumscribed Cell-Surface Domains, and Release on Microvesicles

To investigate cellular mechanisms that are responsible for the production and release of proteolytically active MVs from smoke-exposed human macrophages, we focused on the effects of TSE on induction, activation, and membrane accumulation of MMP14, the dominant gelatinase and collagenase we found on macrophage MVs (Figure 1). Tobacco smoke exposure of human THP-1 macrophages (Figure 2A) and primary hMDMs (Figure 2B) significantly increased the cellular content of both inactive pro-MMP14 and active mature MMP14 in a time-dependent manner, which by 20 hours exceeded a sevenfold induction in the primary cells. Dose-dependent induction of cellular MMP14 by TSE is shown in Supplemental Figure S1. Importantly, the majority of human macrophage MMP14 after 20 hours of smoke exposure was the active, mature form (Figure 2, A and B, and Supplemental Figure S1).

To document MMP14 display on the surface of TSE-exposed human macrophages, we used fluorescent confocal microscopy. These images demonstrate a massive induction of cell-surface MMP14 on smoke-exposed THP-1 macrophages compared to the control cells, which had essentially no detectable MMP14 on their surface (Figure 2C). These results confirm and extend our findings with immunoblots of whole-cell homogenates (Figure 2A). Macrophages abnormally linger within atherosclerotic plaques[47–50] and

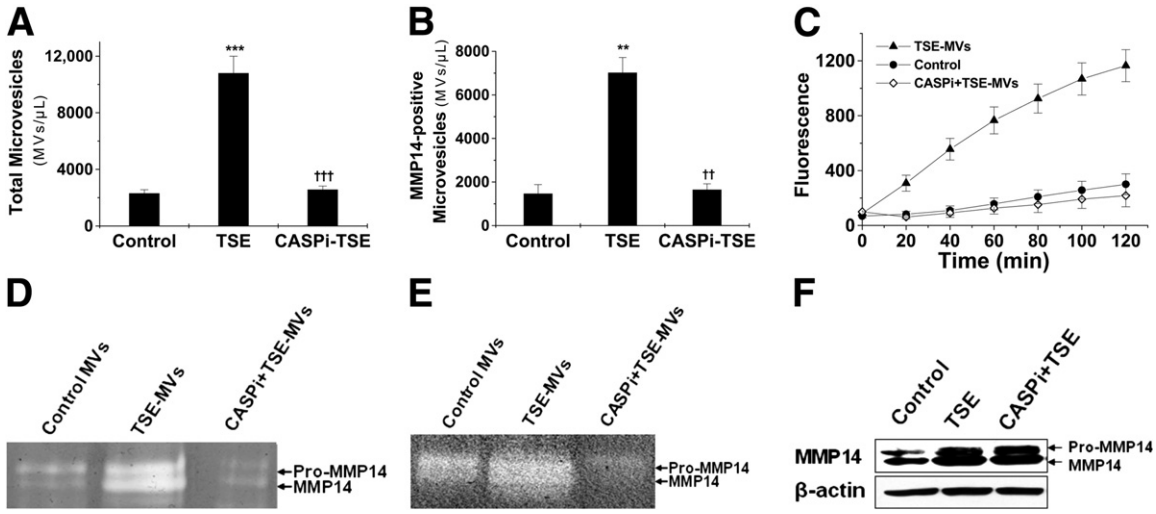

Figure 3 Apoptosis is required for the generation of proteolytically active, MMP14-positive microvesicles from TSE-exposed human macrophages. **A** and **B**: THP-1 macrophages were pre-incubated for 1 hour without (Control, TSE) or with 50 μmol/L Z-VAD-FMK, a pan-caspase inhibitor (CASPi), and then supplemented with buffer (Control) or 2.5% TSE, as indicated, followed by an additional 20-hour incubation. Displayed are the concentrations of total (**A**) and MMP14-positive (**B**) MVs in conditioned media, as assessed by flow cytometry. $P < 0.01$ by analysis of variance; $^{**}P < 0.01$ and $^{***}P < 0.001$ for experimental versus control values; $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ for CASPi-TSE versus TSE values (Student-Newman-Keuls test). **C**: Representative kinetic curves for the cleavage of fluorogenic substrate 1 by MVs isolated from the same conditioned media (shown in **A** and **B**). $P < 0.001$ by analysis of variance at each time point after 20 minutes, but the only values significantly different from the other two groups came from TSE-MVs ($P < 0.01$, Student-Newman-Keuls test). **D** and **E**: Representative gelatin (**D**) and native collagen (**E**) zymography of MVs isolated from the same conditioned media as (shown in **A**–**C**). **F**: Representative immunoblots of total MMP14 content of THP-1 macrophages that were treated (shown in **A**–**E**).

within emphysematous lung tissues[13,45,46]; induction of active MMP14 on their cell surface by tobacco smoke could contribute to extracellular matrix destruction in these settings.

Our confocal images show a remarkable concentration of cell-surface MMP14 in small, circumscribed domains rich in exteriorized PS, a marker of MV generation (Figure 2C). These domains exhibit approximately the same size as MVs (approximately 1 μm) and resemble published images of microvesicle release.[51,52] Thus, smoke-exposed macrophages preferentially localize MMP14 into nascent plasma membrane blebs, presumably as part of the export of this molecule onto MVs. Next we used flow cytometry with the same two stains for MMP14 and exteriorized PS, along with size gating by forward scatter, to analyze the conditioned medium of THP-1 macrophages (Figure 2, D–F) and

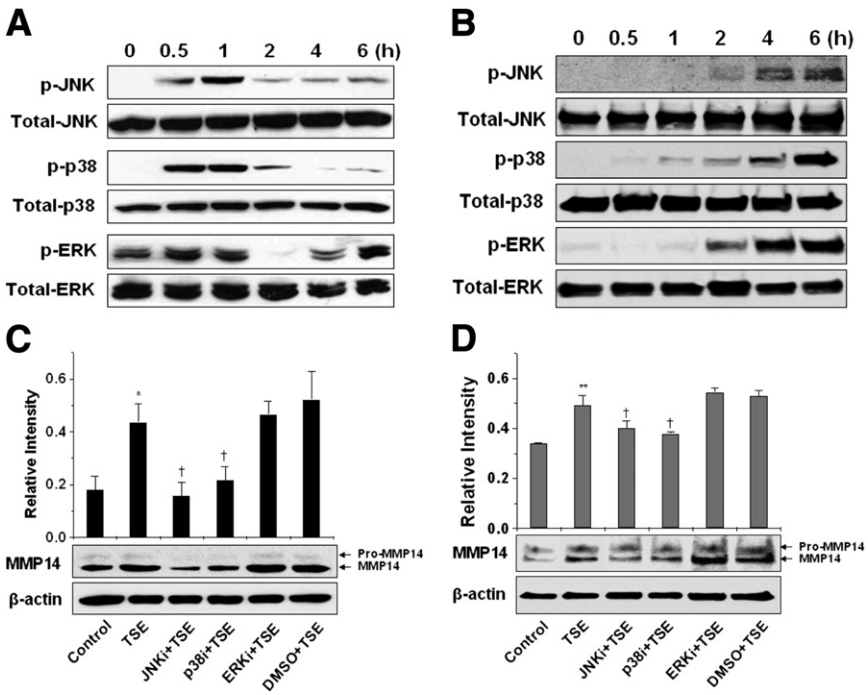

Figure 4 Activation of the JNK and p38 MAPKs is required for TSE-induced expression of MMP14 by human macrophages. **A** and **B**: Representative immunoblots of phosphorylated (p) and total MAPKs (JNK, p38, ERK) after the indicated periods of exposure of THP-1 macrophages (**A**) and primary hMDMs (**B**) to 2.5% TSE. All cells were harvested simultaneously; TSE was added at the indicated times before harvest. **C** and **D**: Quantifications and representative immunoblots of total MMP14 content of THP-1 macrophages (**C**) and primary hMDMs (**D**) that were pretreated for 1 hour without (control, dimethyl sulfoxide) or with the indicated MAPK inhibitors (i), followed by 20-hour exposure to 0% (control) or 2.5% TSE. These quantifications come from three to five independent experiments. $P < 0.01$ by analysis of variance; $^{*}P < 0.05$ and $^{**}P < 0.01$ for experimental versus control values; $^{\dagger}P < 0.05$ for values from cells treated with supplemented TSE versus TSE alone (Student-Newman-Keuls test).

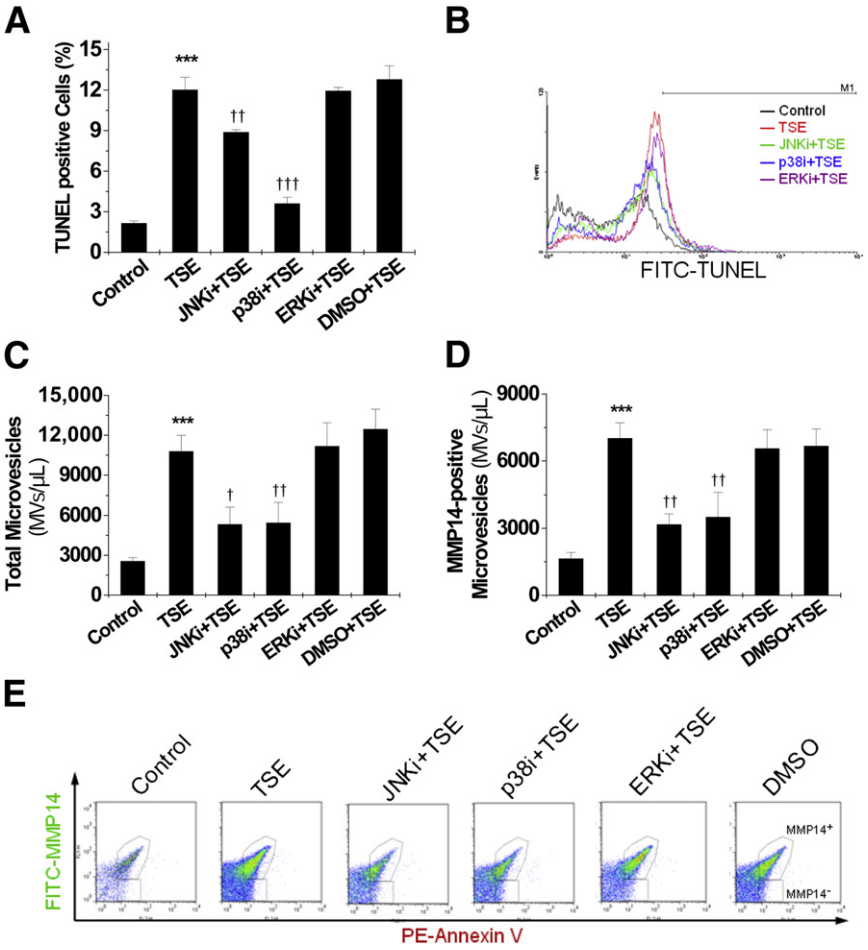

Figure 5 Activation of the JNK, especially p38 MAPKs is required for TSE-induced apoptosis and the generation of total and MMP14-positive MVs from human macrophages. **A**: Quantifications of TUNEL-positive THP-1 macrophages by flow cytometry after the same treatments (as in Figure 4C) (ie, with or without the indicated MAPK inhibitors, with or without TSE). **B**: Representative histograms of TUNEL staining of hMDMs after the same treatments (as in Figure 4C). Displayed are events (*y* axis) versus FITC-TUNEL staining (*x* axis). Note that the *x* axis scale is logarithmic. **C** and **D**: Concentrations of total (**C**) and MMP14-positive (**D**) MVs in conditioned media, assessed by flow cytometry, from THP-1 macrophages after the same treatments (as in Figure 4C). Quantifications in each panel come from four to six independent experiments. **E**: Representative dot plots from flow cytometry of conditioned medium from primary hMDMs after the same treatments (as in Figure 4C). Displayed are signals from FITC-labeled anti-MMP14 antibodies (*y* axis) versus phycoerythrin-annexin V (*x* axis). In each dot plot, events within the enclosed regions represent $MMP14^{+}$-MVs and MMP14-MVs, as indicated. $P < 0.001$ by analysis of variance; $^{***}P < 0.001$ for experimental versus control values; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ for values from cells treated with supplemented TSE versus TSE alone (Student-Newman-Keuls test).

primary hMDMs (Figure 2, G and H) after a 20-hour exposure to 0% (controls) or 2.5% TSE. These results show that tobacco smoke substantially increased the production of total MVs (Figure 2, D, E, and G) and MMP14-positive MVs (Figure 2, D, F, and H). Of note, the majority of smoke-induced MVs carried detectable amounts of MMP14 (comparing the enumerations in Figure 2F and 2H versus 2E and 2G), indicating widespread proteolytic activity. This result is also consistent with the images in Figure 2C, in which nearly all annexin V-positive regions of the cell surface were also stained for MMP14.

Apoptosis Is Required for the Generation of Proteolytically Active MMP14-Positive Microvesicles from TSE-Exposed Human Macrophages

Apoptosis is a major mechanism for microvesicle generation.[3–7] Our confocal images show that TSE induces exteriorization of PS on the cell surface, which can be a sign of early-stage apoptosis, although as previously noted, the binding of annexin V was concentrated in small circumscribed domains, indicating only incomplete loss of membrane asymmetry (Figure 2C). Consistent with prior literature in other cell types,[53–55] including human monocytes,[5] we found that exposure of THP-1 macrophages to TSE for 20 hours induced dose-dependent TUNEL staining, a definitive marker of late-stage apoptosis (Supplemental Figure S2, A and B). Pretreatment of these macrophages with a caspase inhibitor significantly attenuated TSE-induced apoptosis (Supplemental Figure S2B), and the inhibitor almost completely blocked smoke-induced generation of total and MMP14-positive MVs (Figure 3, A and B). More importantly, caspase inhibition abolished TSE-induced production of MVs with proteolytic activities against fluorogenic substrate I (Figure 3C), gelatin (Figure 3D), or native collagen (Figure 3E). Similar to our prior results on the regulation of tissue factor,[5] caspase inhibition did not affect the induction of cellular MMP14 expression by TSE (Figure 3F), implying an effect solely on MV release. Thus, inhibition of apoptosis in this context does not affect the induction of abundant amounts of mature MMP14 in the cells (Figure 3F), but dramatically inhibits the release of this MMP14 on MVs (Figure 3).

Activation of the JNK, Especially the p38 MAPKs, Is Required for TSE-Induced Expression of MMP14 by Human Macrophages, Apoptosis, and the Generation of Proteolytically Active, MMP14-Positive MVs

Next we focused on mechanisms for smoke-induced expression of MMP14 by human macrophages. Consistent with prior

literature in other cell types,[56,57] including human monocytes,[5] we found that exposure of THP-1 macrophages to TSE for 20 hours increased the phosphorylation of three major MAPKs (ie, JNK, p38, and ERK) (Figure 4A). The biphasic ERK response in Figure 4A was reproducible and resembles ERK activation in some,[58,59] but not all,[5] other circumstances. In primary hMDMs, TSE also activated all three MAPKs, but the activation occurred later than in THP-1 macrophages and persisted to the 6-hour time point (Figure 4B). Pretreatment of the THP-1 macrophages with either the JNK inhibitor SP600125 or the p38 inhibitor SB202090 completely blocked the ability of smoke to induce MMP14 expression, indicating that activation of both kinases is required (Figure 4C). The ERK inhibitor (U0126) had no detectable effect, indicating pathway specificity (Figure 4C). A similar pattern was evident for the effects of these MAPK inhibitors on TSE-induced expression of MMP14 by primary hMDMs, indicating roles for JNK and p38 but not ERK (Figure 4D). The involvement of MAPK signaling pathways in expression of MMP14 by macrophages had not been previously reported, although several prior studies found a role for JNK, but not ERK, in MMP14 induction in other cell types exposed to other stimuli, consistent with our current results.[45,60,61]

Next we examined the role of MAPKs in TSE-induced apoptosis and the generation of MMP14-positive MVs from human macrophages. Our recent work in human monocytes showed that tobacco smoke-induced apoptosis and apoptotic MV generation is mediated through ERK in those cells.[5] Unexpectedly, pretreatment of human THP-1 macrophages with the JNK or p38 inhibitor provided significant protection from TSE-induced apoptosis, whereas the ERK inhibitor had no effect (Figure 5A and Supplemental Figure S3A). A similar pattern was evident for the effects of these MAPK inhibitors on TSE-induced apoptosis of primary hMDMs (Figure 5B). Likewise, inhibition of JNK or p38 inhibited smoke-induced production of total and MMP14-positive MVs from both THP-1 macrophages (Figure 5, C and D) and primary hMDMs (Figure 5E), again indicating a need for activation of both of these kinases for these effects to occur in macrophages. Inhibition of ERK, however, had no detectable effect on any of these parameters, demonstrating pathway and cell-type specificity (Figure 5, A–E, and Supplemental Figure S3, A and B), consistent with the extensive changes that occur during monocyte-to-macrophage differentiation.[62–65]

Finally, we tested the role of MAPKs in the generation of proteolytic MVs from smoke-exposed human macrophages. Pretreatment of THP-1 macrophages with MAPK inhibitors was followed by exposure to TSE for 20 hours and then ultracentrifugal isolation of MVs from conditioned media. Inhibition of JNK or p38, but not ERK, completely blocked TSE-induced production of MVs with proteolytic activities against fluorogenic substrate I (Figure 6A), gelatin (Figure 6B), and native collagen (Figure 6C). These results indicate that activation of the JNK and p38 signaling pathways is responsible for the TSE-induced generation of proteolytically active MVs from human macrophages through activation of MMP14 expression, apoptosis, and membrane blebbing.

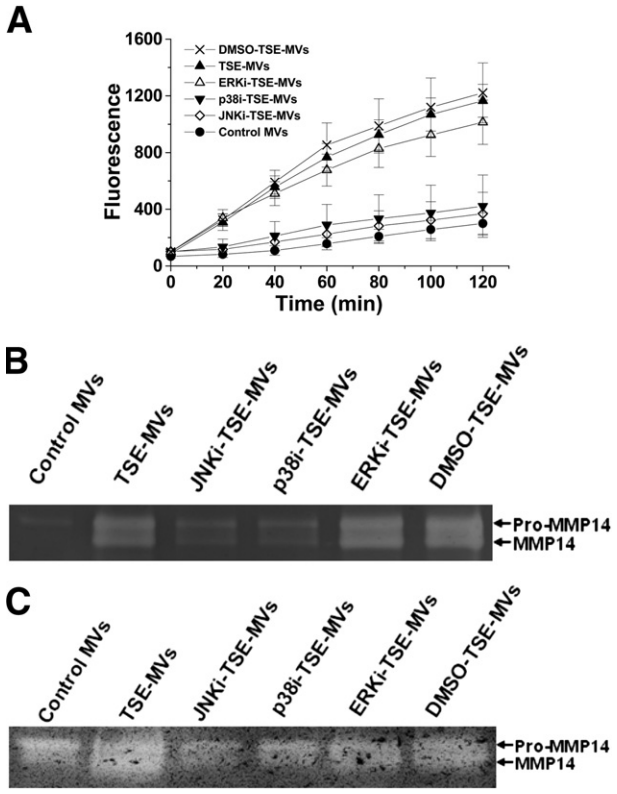

Figure 6 Activation of the JNK and p38 MAPKs is required for TSE-induced generation of proteolytically active MVs from human macrophages. **A:** Human THP-1 macrophages were pre-incubated for 1 hour without [control, dimethyl sulfoxide (DMSO)] or with the indicated MAPK inhibitors, then supplemented with buffer (Control) or 2.5% TSE, followed by additional 20-hour incubation. Displayed are kinetic curves for the cleavage of fluorogenic substrate 1 by MVs isolated from the conditioned media of these cells. $P < 0.001$ by analysis of variance at each time point after 20 minutes. Each member of the upper cluster of values (DMSO-TSE MVs, TSE MVs, and ERKi-TSE MVs) was significantly different from each member of the lower cluster of values (p38i-TSE MVs, JNKi-TSE MVs, and Control MVs), $P < 0.05$, by the Student-Newman-Keuls test. **B** and **C**: Representative gelatin (**B**) and collagen (**C**) zymography of MVs isolated from the conditioned medium of THP-1 macrophages after the same treatments (as shown in **A**). **A**–**C** are representative from at least three independent experiments. ERKi, ERK inhibitor; JNKi, JNK inhibitor; p38i, p38 inhibitor.

Discussion

Our study demonstrates that exposure of human macrophages to tobacco smoke extract induces the release of potently proteolytic MVs. We found that smoke-induced macrophage MVs carry substantial gelatinolytic and collagenolytic activities that can be attributed, surprisingly, predominantly to a single transmembrane protease of the MMP superfamily — namely, MMP14. Based on our biochemical and morphological studies, the production of these MVs relies on a series of dynamic, regulated steps that include activation of the JNK and p38 MAPKs, MAPK-dependent induction of cellular MMP14, cleavage of pro-MMP14 into its active mature form, a remarkable accumulation of MMP14 into nascent plasma membrane blebs, and finally, caspase- and MAPK-dependent apoptosis and apoptotic blebbing.

MMP14 is of particular relevance to unstable atherosclerosis, given the ability of the enzyme to disrupt collagen within murine plaques *in vivo*[43] and its abundance within human atheromata,[41] particularly in macrophages within the rupture-prone shoulders of advanced plaques[47] Of note, apoptotic macrophages have been found at sites of plaque rupture in sudden coronary death,[66] consistent with the production of biologically active MVs. Moreover, MMP14 has been reported to activate the gelatinase MMP2[42] and destroy apolipoprotein E,[67] a protein with anti-atherogenic properties when expressed by macrophages.[68] In addition, emphysematous lungs exhibit upregulation of MMP14 in alveolar macrophages,[13,45,46] a cell type directly exposed to cigarette smoke and prone to apoptosis.[53] Taken together, TSE-induced expression of MMP14 by human macrophages and then its release on collagenolytic MMP14-positive MVs may contribute to the conspicuous instability of atherosclerotic plaques and the destruction of pulmonary connective tissue in smokers. High levels of MMP14 on cells and on MVs may also contribute to other complications of smoking, such as loss of dermal collagen.

Each activated or apoptotic cell can release many MVs, and owing to their small size, the MVs often survive longer and diffuse more readily than their parental cells.[7,69,70] Overall, the ability of TSE to induce expression of MMP14 by human macrophages and then the activation and export of this molecule on MVs may be a particularly potent pathway to damage extracellular matrix in tissues and organs of individuals exposed to tobacco smoke.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.01.035*.

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